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<b>(54) Title:</b> USE OF LEPTIN ANTAGONISTS FOR TREATING INSULIN RESISTANCE IN TYPE II DIABETES  <b>(57) Abstract</b>  The invention relates to pharmaceutical agents containing leptin antagonists for treating Type II diabetes. One leptin antagonist is based on a murine leptin fragment and comprises amino acids 116 to 167 or 116 to 166. Methods of treating Type II diabetes are also disclosed.		

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## Use of leptin antagonists for treating insulin resistance in Type II diabetes

### Background of the Invention

The present invention relates to the use of leptin antagonists for treating insulin resistance in Type II diabetes and to a pharmaceutical for treating such resistance.

Diabetes is one of the most frequently occurring metabolic diseases in industrialized countries. There are some 110 million diabetics world-wide; while approx. 10 million of these are Type I diabetics, the overwhelming majority (approx. 100 million) are Type II diabetics. The disease is caused by faulty regulation of glucose metabolism. In Type I diabetes, failure of the  $\beta$  cells in the pancreas results in insulin no longer being formed. This lack of insulin leads to an increase in blood glucose and, if not treated by supplying insulin, to ketoacidosis, diabetic coma and death of the patient. In Type II diabetics, the causal relationships are different and are characterized by the initial development of insulin resistance, i.e. diminution in the ability of the cells to respond adequately to insulin. Excessive weight and lack of physical activity, in particular, are regarded as being responsible for inducing insulin resistance. The latter condition is not noticed initially since it is offset by an increased secretion of insulin. However, the continuing insulin resistance leads, in a process extending over many years, to failure of the endogenous compensation mechanism and consequent development of Type II diabetes. While diet and physical activity can delay this sequence of events, they are frequently unable to prevent manifestation of the disease. Medicinal intervention is then required in order to control the blood glucose adequately.

It is of crucial importance for the long-term success of the therapy that the blood glucose be maintained as narrowly as possible within the physiological range. It is the current view that glucose levels which have been elevated for decades, as are found in poorly controlled diabetics (both Type I and Type II diabetics), make an important contribution to late complications in diabetes. These late complications

constitute, in particular, blood vessel damage which leads to kidney diseases, loss of sight and cardiovascular diseases. This so-called late damage is an important factor contributing to mortality in diabetics.

In 1994, a new hormone, leptin, was described which is formed in fat cells and which is lacking in genetically overweight mice (ob/ob mice) (Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J.M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature* 372, 425-432.). Human leptin and murine leptin are to a large extent identical. Injecting ob/ob mice with recombinantly prepared leptin leads to a reduction in nutrient intake and to a decrease in weight (Pelleymounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T., and Collins, F. (1995). Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* 269, 540-543.). There has so far been no indication that mutations in the ob gene might be responsible for the frequent occurrence of obesity in humans (approx. 30% of the population is markedly overweight in the USA). Systematic investigations have demonstrated that serum levels of leptin are increased in obese humans as they are in various animal models of obesity (Dagogo-Jack, S., Fanelli, C., Paramore, D., Brothers, J., and Landt, M. (1996). Plasma leptin and insulin relationships in obese and nonobese humans. *Diabetes* 45, 695-698; Considine, R.V., Sinha, M.K., Heiman, M.L., Kriauciunas, A., Stephens, T.W., Nyce, M.R., Ohannessian, J.P., Marco, C.C., McKee, L.J., Bauer, T.L., and Caro, J.F. (1996). Serum immunoreactive leptin concentrations in normal-weight and obese humans. *N. Engl. J. Med.* 334, 292-295.). For this reason, it is assumed that leptin is a feedback signal which informs the brain of the quantity of energy which is stored in the fat tissue. According to this assumption, it is then the function of the brain to decrease feed intake by inhibiting appetite, on the one hand, and to stimulate basal metabolism on the other. In human obesity, this regulatory circuit appears to be interrupted.

In addition to this, it is assumed that leptin also acts directly on tissues outside the brain.

Three studies relating to the direct effect of leptin on isolated cells have so far been published:

Kroder et al. (Kroder, G., Kellerer, M., and Häring, H. (1996) *Exp. Crin. Endocrin. Diabetes* 104 Suppl. 2, 66 (Abstract)) made the assumption that leptin establishes a connection between insulin resistance and obesity and report that leptin decreases insulin-induced phosphorylation of the insulin receptor and of insulin receptor substrate 1 (IRS-1) in rat 1 fibroblasts which are overexpressing the human insulin receptor. The extent to which leptin also exerts an influence on the end points of the insulin effect, for example stimulation of glucose transport or glycogen synthase, was not investigated or discussed.

It has been demonstrated that sensitivity to lipogenic hormones (dexamethasone and insulin) is decreased in transformed 30A5 preadipocytes which are overexpressing leptin (Bai, Y. L., Zhang, S. Y., Kim, K. S., Lee, J. K., and Kim, K. H. (1996) *J. Biol. Chem.* 271, 13939-13942). Fatty acid synthesis and the synthesis of neutral lipids were decreased by leptin overexpression even in the non-stimulated state. While control cells exhibited a marked increase in the rate of lipid synthesis after the cells had been treated with dexamethasone or insulin, or a combination of the two hormones, cells which were overexpressing leptin were hardly stimulated at all under these circumstances. In addition to this, an investigation was also undertaken of the inhibition of glycerophosphate dehydrogenase activity and of acetyl CoA carboxylase expression which occurs after treating the cells with a combination of dexamethasone and insulin. It was found that it was not possible to stimulate leptin-expressing cells. The effects which were observed indicate that leptin suppresses lipid metabolism in a general manner. There is no mention of any possible connection between obesity and insulin resistance.

In another model system, i.e. C<sub>2</sub>C<sub>12</sub> mouse myotubes, it was found that leptin exhibits insulin-like effects (Berti, L., Kellerer, M., and Häring, H. (1996) *Diabetologia* 39 Suppl. 1, A59 (Abstract)). This study reports that both glucose

transport and glycogen synthesis are stimulated by leptin. These findings conflict with those reported by the other authors and the results which are presented here. They possibly involve an effect which is specific for this cell type.

In our investigations into the effect of leptin on isolated rat adipocytes, a model system for fat tissue, it has now been found, surprisingly, that the insulin sensitivity of important metabolic pathways of the fat cell such as stimulation of lipogenesis, of glucose transport and of glycogenesis, is drastically reduced (Example 4) whereas the basal values remain unaffected. The same applies to the inhibition of isoproterenol-stimulated lipolysis. Glucose transport in isolated rat adipocytes is stimulated approximately 14-fold by adding insulin (10 nM). This capacity to be stimulated is reduced in a dose-dependent manner by preincubating with leptin at different concentrations for 15 hours. Leptin desensitizes the cells, i.e. an insulin resistance is produced. Dose/effect curves for insulin at different leptin concentrations (Example 5) demonstrate that the concentrations at which effects can already be detected in vitro, both as regards insulin (0.1 - 0.2 nM) and as regards leptin (0.5 - 1 nM), are within the physiological range (Dagogo-Jack et al., 1996; Considine et al., 1996). Higher leptin levels (2-4 nM) (Dagogo-Jack et al., 1996; Considine et al., 1996), are found in obese humans, so that it is possible that the insulin effect is more strongly impaired in these subjects. The conclusion therefore suggests itself that chronically elevated leptin, as can be seen in obese subjects, leads to insulin resistance. As already explained above, insulin resistance is an important factor in the pathogenesis of Type II diabetes.

It is therefore an object of the invention to provide novel leptin antagonists that may be formulated in pharmaceutical compositions. It is another object of the invention to provide methods of treating Type II diabetes and other insulin-related disorders.

The invention consequently relates to the use of leptin antagonists, in particular those which are derived from leptin itself, for preparing a pharmaceutical for use in Type II diabetes. The leptin antagonists for this use are described in more detail below.

**Summary of the Invention**

According to a first object of the invention, pharmaceutical compositions are provided which comprise an antagonist of leptin. According to this same object, pharmaceutical compositions are disclosed which comprise leptin antagonists which are derived from leptin. Further according to this object, pharmaceutical compositions are revealed which comprise a leptin antagonist that is a soluble leptin receptor, or a derivative thereof.

According to a second object of the invention, methods are provided which utilize the inventive pharmaceutical compositions in the treatment of Type II diabetes. Also according to this object, methods are provided for restoring or amplifying the physiological effect of insulin.

**Brief Description of the Drawings**

- Table 1: Inhibition of insulin-induced glucose transport by leptin in rat adipocytes. The experiment is described in detail in Example 8.
- Table 2: 2-Deoxyglucose uptake by rat adipocytes as a function of the leptin concentration. The experiment is described in detail in Example 9.
- Table 3: Antagonism of the leptin effect by leptin fragment 116-167 in relation to insulin-induced glucose transport in rat adipocytes. The experiment is described in detail in Example 10.
- Table 4: The amino acid sequence of human leptin. The two cysteines are linked by a disulfide bridge.
- Table 5: The amino acid sequence of murine leptin. The two cysteines are linked by a disulfide bridge.
- Table 6: The amino acid sequence of fragment 116-167 of murine leptin. The two cysteines are linked by a disulfide bridge.

**Detailed Description of the Invention**

The invention which is presented here is directed towards compositions and methods for reducing, or completely eliminating, insulin resistance by inhibiting the effect of leptin. For this purpose, use can be made of peptides which act as leptin antagonists and which lead, in isolated fat cells in vitro, to abolition of leptin-induced insulin resistance. These peptides are consequently suitable for the therapy of insulin resistance, preferably in obese patients.

**Leptin Antagonists**

Leptin antagonists according to the invention specifically include peptide antagonists. The said peptides are derived from leptin fragments and may be obtained, for example, by chemically or enzymically cleaving intact leptin (e.g. with lysyl endopeptidase, trypsin, endo-Arg C or cyanogen bromide) or be prepared by being expressed, directly or as a fusion protein, in microorganisms. In connection with preparing the peptides in microorganisms, there is no compulsion to rely on the presence of natural cleavage sites when selecting the fragments which are to be expressed. Human and animal leptin, for example rat, mouse, pig or humanoid ape leptin, are suitable for deriving the peptides.

One suitable exemplary peptide extends from amino acid 116 to amino acid 167 or from amino acid 116 to amino acid 166 (Table 6, SEQ ID NO.: 4) in accordance with the sequence which has been published in Zhang et al., (1994) (Examples 3 and 6). In Example 6, adipocytes were incubated for approx. 15 hours in the presence of 10 nM leptin and different concentrations of the antagonistic 116-167 leptin fragment. The cells were then stimulated with 5 nM insulin. In this experiment, it was found that increasing quantities of the antagonist lead to restoration of the capacity to be stimulated by insulin and no resistance develops in the presence of high concentrations of the leptin antagonist. Thus, using these and similar assays, one skilled in the art readily may ascertain the antagonistic properties of any leptin antagonist that would be useful according to the present invention.



In addition to this, analogues of the antagonistic leptin fragments can also be used in which one or more amino acids is/are replaced or deleted. Preferable replacements are conservative amino acid substitutions. Such conservative substitutions include charged-charged, polar-polar and hydrophobic-hydrophobic amino acid substitutions. For example, one or more aspartate residues can be replaced by glutamate residues and/or vice versa and/or one or more leucine residues can be replaced by isoleucine residues and/or vice versa. Other substitutions and deletions may rationally be made based on steric and structural considerations, such as amino acid size and propensity for helix making or breaking.

Molecular biological and biotechnological methods can be used to alter and optimize the antagonistic properties of the said peptides in a specific manner. In addition to this, the peptides can be modified chemically, for example by means of acetylation, carbamoylation, formylation, biotinylation, acylation, or derivatization with polyethylene glycol or hydrophilic polymers, in order to increase their stability or modulate their plasma half-life and pharmacokinetics.

Antibodies against leptin, in particular their leptin-binding domains, are also suitable as leptin antagonists for the said purpose. In addition to this, soluble leptin receptors and/or leptin receptor fragments, and fusions thereof with other proteins (e.g. the IgG Fc region), are also suitable. Like the peptide antagonists, any leptin antagonist which is a protein may be altered by molecular biological means. Similarly, these antagonists may be prepared by being expressed, directly or as a fusion protein, in microorganisms or any number of standard expression systems.

### **Pharmaceutical Compositions**

The invention furthermore relates to a pharmaceutical which comprises the leptin antagonists which are described in this patent application.

The pharmaceuticals can be used, for example, in the form of pharmaceutical preparations which can be administered orally, for example in the form of tablets, coated tablets, hard or soft gelatin capsules, solutions, emulsions or suspensions. They can also be administered rectally, for example in the form of suppositories, or

parenterally, for example in the form of injection solutions. The pharmaceuticals can also be administered by way of the mucous membranes of the nose, of the mouth or of the lung. In order to produce pharmaceutical preparations, these compounds can be worked into therapeutically inert, organic and inorganic excipients. Lactose, corn starch or derivatives thereof, talc and stearic acid or salts thereof are examples of such excipients for tablets, coated tablets and hard gelatin capsules. Water, polyols, sucrose, invert sugar and glucose are suitable excipients for preparing solutions. Water, alcohols, polyols, glycerol and vegetable oils are suitable excipients for injection solutions. Vegetable and hardened oils, waxes, fats and semi-liquid polyols are suitable excipients for suppositories. The pharmaceutical preparations can also comprise preservatives, solvents, stabilizers, wetting agents, emulsifiers, sweeteners, dyes, flavorants, salts for altering the osmotic pressure, buffers, coating agents, antioxidants and, where appropriate, other therapeutic active compounds.

Oral administration and injections are preferred. For injection, the novel leptin antagonists are formulated in a liquid solution, preferably in a physiologically acceptable buffer such as Hank's solution or Ringer's solution. However, the novel leptin antagonists can also be formulated in solid form and be dissolved or suspended prior to use.

Typical formulations contain a therapeutically beneficial amount of a leptin antagonist. A therapeutically beneficial amount may be the same as a therapeutically effective amount, as discussed below. Additionally, a therapeutically beneficial amount may be a unit dose, which either alone or in multiples may be used to provide a therapeutically effective amount of leptin antagonist. Thus, a therapeutically beneficial amount will rely, inter alia, on the nature of the disorder being treated.

### **Methods of Treatment**

The methods of the invention are useful in treating any disorder in which the action of leptin is implicated. In view of the present disclosure and the observation that leptin inhibits some of the physiological activities of insulin, the inventive methods are particularly useful in the treatment of disorders involving perturbations of insulin

activity, and especially Type II diabetes. Such perturbations in insulin activity include alterations in lipogenesis, glucose transport, glycogenesis and lipolysis. Accordingly, the inventive methods include methods for treating Type II diabetes and methods for restoring or amplifying the physiological effects of insulin.

A typical method entails administering to a patient in need of treatment a therapeutically effective amount of a leptin antagonist. A patient will be in need of treatment when suffering from a disorder in which the action of leptin is implicated. Treatment is especially indicated when a patient is suffering from Type II diabetes. Treatment is also indicated when a patient is suffering from a disorder characterized by a perturbation in insulin activity, such as alterations in lipogenesis, glucose transport, glycogenesis and lipolysis. In disorders where such a perturbation is implicated, methods for restoring or amplifying the physiological effect of insulin are useful.

A therapeutically effective amount will depend, for example, on the nature of the disorder being treated, the route of administration, the particular characteristics of the antagonist chosen, and especially the judgement of the attending clinician. A therapeutically effective amount generally is an amount sufficient to effect treatment of the disease targeted or to accomplish the stated goal of the method, for example, restoring or amplifying the physiological effect of insulin. Ultimately the therapeutically effective amount will depend on the clinically determined efficacy and toxicity of each leptin antagonist. Such determinations are routinely made and well within the ordinary skill of the clinician.

The term "treating" in its various grammatical forms in relation to the present invention refers to preventing, curing, reversing, attenuating, alleviating, minimizing, suppressing or halting the deleterious effects of a disease state, disease progression, disease causative agent or other abnormal condition.

The doses which are preferred for systemic administration are from approx. 0.01 mg/kg to approximately 50 mg/kg of body weight and per day.

The invention is clarified below by means of the Tables and Examples without being restricted thereto.

## Examples

### Example 1: Cloning murine leptin

**RNA isolation** - Epididymal fat pads are removed from adult mice and shock-frozen in liquid nitrogen. 1 g of fat tissue is ground in a mortar under liquid nitrogen, after which 15 ml of a 5 M solution of guanidinium thiocyanate in 50 mM Tris (pH 7.5), 10 mM EDTA and 0.1 M DTT are added and the whole is homogenized vigorously in order to obtain a fine dispersion. After the tissue particles have been completely dissipated, 10 g of solid CsCl are added and the mixture is stirred at room temperature. After adding 10 ml of H<sub>2</sub>O, 9 ml of a 5.7 M solution of CsCl are overlaid with 25 ml of this solution in a centrifuge tube. After centrifuging for 15 hours in an SW28 rotor at 25,000 rpm (18°C), the tube is deep-frozen in liquid nitrogen and the bottom quarter of the tube is cut off using a hot scalpel blade; the frozen contents are taken out and the RNA pellet is scraped off their bottom end. The RNA is dissolved and then precipitated with ethanol.

**cDNA synthesis** - In a mixture, 1 µg of total RNA from fat tissue and 1 µg of the specific primer oligonucleotide 5'-GAATGCAGAATAAATAAATA (SEQ ID NO.: 1; Zhang et al., 1994) are dissolved in 10 µl of H<sub>2</sub>O and are then heat-denatured and incubated at 65°C for 5 min. After adding 0.5 µl of RNase inhibitor, in each case 5 nmol of dNTP and 0.5 µl of AMV reverse transcriptase (Boehringer Mannheim), the mixture is incubated at 42°C for 1 h. After that the cDNA is made up to 200 µl with H<sub>2</sub>O and stored at -20°C.

**PCR** - 3 µl of the specifically primed cDNA are amplified with 0.5 µg each of the two primers 5'-GAAAGAAGGATCCAGTGCCTATCCAGAAAGTCCA (SEQ ID NO.: 2) and 5'-GGAGAGAAGCTTGAGGGAGAGAAATGAATGATGG (SEQ ID NO.: 3; Zhang et al., 1994) and 2.5 U of Taq polymerase (Perkin Elmer) for 30 cycles in the

reaction buffer recommended by the manufacturer (1.5 mM MgCl<sub>2</sub>, 200 µM dNTPs in 100 µl). Each cycle comprises 1 min at 94°C, 1 min at 55°C and 2 min 72°C.

Ligation - The specifically amplified PCR product (583 bp) from a PCR preparation is cleaved, in each case at 37°C for 2 h and under buffering conditions which accord with the manufacturer's instructions, with the restriction enzymes BamHI and HindIII (Boehringer Mannheim), after which the 564 bp fragment is purified by electrophoresis and isolated. It is incubated, at 30°C for 2 h and in 20 µl, together with 0.1 µg of BamHI- and HindIII-cleaved vector pQE31 (Qiagen) and 20 U of T4 DNA ligase (New England Biolabs).

Cloning - 5 µl of the ligation mixture are kept on ice for 30 min together with 100 µl of transformation-competent E.coli cells of the HB101 strain and the mixture is then swirled gently for 5 min in a water bath at 37°C. Following the addition of 0.9 ml of nutrient medium containing 10 mM MgCl<sub>2</sub>, the mixture is shaken at 37°C for 1 h. 100 µl volumes of this mixture are in each case plated onto ampicillin-containing (100 µg/ml) agar plates.

Identification of the clones - The clones which have grown overnight at 37°C are inoculated into 2 ml-volume liquid cultures containing ampicillin, cultured to stationary phase and then centrifuged down. The cells are suspended in 0.1 ml of 25 mM Tris (pH 8), 50 mM glucose, 10 mM EDTA and lysozyme (2 mg/ml) and, after having been incubated at room temperature for 5 min, are lysed by adding 0.2 ml of 0.2 M NaOH, 1% SDS. The chromosomal DNA is precipitated by adding 150 µl of 3 M Na acetate/acetic acid (pH 5.2) and centrifuged down for 5 min at 4°C (10,000 rpm in a Sigma 2MK). The plasmid DNA is precipitated with 2.5 volumes of ethanol, centrifuged down (see above) and, following an ethanol wash, taken up in 100 µl of H<sub>2</sub>O; 10 µl of RNase solution (10 mg/ml) are then added.

The plasmid DNA is digested with restriction enzymes (BglI, XhoI + PvuII; Boehringer Mannheim) in accordance with the manufacturer's instructions, and the resulting DNA fragments are measured against marker DNA in an agarose gel following electrophoresis and staining with ethidium bromide. Clones which have the correct fragment pattern are examined in the same way using the restriction enzyme

AflIII (New England Biolabs) for the presence of the glutamine 49 residue.

The identity of in each case one E. coli clone containing Gln49 (pQEob3-9) and one not containing Gln49 (pQEob3-4) was confirmed by means of DNA sequencing. The production of recombinant leptin containing the presequence MetArgGlySer(His)6ThrAspPro (from the vector pQE31) followed by amino acids 22 to 167 from murine leptin was checked in small cultures.

While both recombinant leptins (with and without Gln49) can be employed in the experiments which are described below, the examples specifically relate to leptin which contains Gln49 and which is obtained by expressing pQEob3-9.

#### Example 2: Preparation of leptin

**Disruption** - The bacteria from a 10 l fermentation are centrifuged at 4800 rpm for 20 min. The sediment is frozen at -20°C and subsequently suspended in lysis buffer (6 M guanidinium chloride, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris/HCl, pH 8) (5 ml of lysis buffer/g of sediment), after which the mixture is stirred at room temperature for one hour and then centrifuged at 4800 rpm for 30 min.

**Ni-NTA chromatography** - 100 ml of Ni-NTA agarose FF (Qiagen, Hilden) are added to crude extract which contains approx. 800 mg of leptin and the whole is stirred at 4°C overnight. The suspension is sucked through a glass column (ø 5 cm) possessing a frit. The column, together with the Ni-NTA agarose which is contained in it, is washed with 300 ml of lysis buffer and then with 100 ml of lysis buffer containing 10 mM imidazole (5 ml/min). Fractional elution of the leptin then takes place by applying a linear gradient of 10 to 200 mM imidazole in lysis buffer (gradient volume: 300 ml at 5 ml/min). The fractions are analyzed by RP-HPLC and those which are of adequate purity and concentration are combined (=Ni-NTA eluate).

**Folding** - The Ni-NTA eluate is diluted with lysis buffer to a concentration of 1 - 3

mg/ml and adjusted to pH 9 with sodium hydroxide solution. After adding  $\beta$ -mercaptoethanol (from 4 to 6 mol of  $\beta$ -mercaptoethanol per mole of leptin), the mixture is incubated at room temperature for 2 hours in a sealed container. For reoxidation and refolding, the solution is poured into 9 times the volume of folding buffer (0.1 M Tris/HCl, pH 9) and the whole is stirred at 16°C for from 16 to 24 hours while admitting air. Any turbidity which appears is centrifuged off (4,000 rpm, 45 min).

**Reversed phase HPLC** - The folding mixture is adjusted to pH 3 with HCl and pumped onto a 2.5 x 30 cm RP column (PLRPS 300 Å, 10  $\mu$ , Polymer Laboratories, Amherst, USA) at the rate of 20 ml/min. The column is subsequently washed with 300 ml of eluent A (0.1% aqueous TFA). Leptin is eluted in 100 minutes (flow rate: 7 ml/min) by applying a gradient of from 25 to 50% eluent B (0.09% TFA in acetonitrile). The fractions are analyzed by means of analytical HPLC. Fractions of adequate purity and concentration are combined (RP pool). The pool is treated with 7 mmol of  $\text{Na}_2\text{HPO}_4/\text{l}$  and adjusted to pH 3 with NaOH, and the solvent is removed on a rotary evaporator. The aqueous leptin solution is then neutralized with NaOH (pH 7.4) and stored at 4°C overnight. Any resulting turbidity is centrifuged off (4,000 rpm, 10 min).

**Gel permeation chromatography** - The neutralized and centrifuged leptin solution is concentrated to 10-15 mg/ml by ultrafiltration and then sterilized by filtration. From 50 to 75 mg are loaded onto a Superdex 75 column (2.6 x 60 cm, Pharmacia, Sweden). PBS (154 mM NaCl, 10 mM sodium phosphate, pH 7.4) is used as the elution buffer, at a flow rate of 3 ml/min. The leptin which has been purified in this way is then filtered once again through a 0.22  $\mu\text{m}$  membrane and stored at -70°C.

#### Example 3: Preparation of the antagonistic 116-167 fragment

1 ml of 1 M Tris/HCl, pH 8, is added to 40 ml of a solution of leptin (1 mg/ml in PBS) and, after 160  $\mu\text{g}$  of lysyl endopeptidase have been added, the leptin is digested at room temperature for 3 h. The mixture is adjusted to pH 3 and fractionated by

RP-HPLC as described in Example 2. The 116-167 fragment is identified by electrospray mass spectrometry (5532 D), freed from solvent as described in Example 2, concentrated and purified by means of gel permeation chromatography.

#### Example 4: Isolation of adipocytes

Adipocytes were prepared from the epididymal fat tissue of male Wistar rats (140-160 g, Hoechst AG breeding station, Kastengrund) by means of digesting with collagenase (Rodbell, 1964, J. Biol. Chem. 239, 375-380), washed twice with KRH (25 mM Hepes free acid, 25 mM Hepes sodium salt, 80 mM NaCl, 1 mM  $\text{MgSO}_4$ , 2 mM  $\text{CaCl}_2$ , 6 mM KCl, 1 mM sodium pyruvate, 0.5% BSA) and once with DMEM (Dulbecco's minimal essential medium), supplemented with 5.5 mM glucose, 20 mM Hepes (pH 7.4), 2% fetal calf serum, 1% BSA, 50 U of penicillin/ml, 10 mg of streptomycin/ml, by means of flotation (800xg, 1 min, in small plastic tubes), and finally diluted to a volume of 20 ml of DMEM/g wet weight of the fat tissue (cell titer: approximately  $2.5 \times 10^5$  cells/ml).

#### Example 5: Primary culture of the adipocytes and incubation with leptin

The adipocytes were incubated, at 37°C for 15-18 h under an atmosphere of 5%  $\text{CO}_2$  and with gentle shaking, in supplemented (see above) DMEM in the presence of 100 nM phenylisopropyladenosine (4 ml of DMEM to 1 ml of cells, cell titer approximately  $5 \times 10^4$  cell/ml, in 50 ml sterile polypropylene tubes) and in the presence or absence of leptin. The adipocytes were subsequently washed three times with cold KRH and adjusted to a cell titer of approximately  $3 \times 10^5$  cells/ml by adding 0.7 ml of glucose-free KRH to the cell layer which remained after the complete removal of the last washing solution. In order to determine the capacity of the adipocytes to be stimulated by insulin, and their sensitivity to insulin, following the primary culture, the washed adipocytes were incubated at 37°C for 20 min in the absence or presence of human insulin (0.02-50 nM final concentration), and glucose transport or lipogenesis was then measured.



#### Example 6: Glucose transport

Glucose transport was measured as the specific uptake of the non-metabolizable glucose analog 2-deoxyglucose (Müller and Wied, 1993, *Diabetes* 42, 1852-1867). 50 µl of the adipocyte suspension in KRH, which suspension had or had not been preincubated with insulin (see above), were incubated, at 25°C for 5 min, with 50 µl of KRH which was supplemented with 2-deoxy-D-[2,6-<sup>3</sup>H]glucose (0.5 µCi, 0.2 mM). The incubation mixtures were transferred to thin soft-plastic centrifuge tubes, each of which already contained 200 µl of dinonyl phthalate oil, and immediately centrifuged (2,000xg, 30 sec). Using special shears, the tubes were severed within the oil layer (in the vicinity of the upper edge) and the upper halves of the tubes, together with the cell layers which had in each case been floated onto the oil layer, were transferred to scintillation vials. After 10 ml of scintillation fluid (water-based) had been added, the radioactivity associated with the cells was measured. In order to correct for 2-deoxyglucose which had become enclosed in the cell interstices, or had diffused into the cells, in a non-specific manner, the radioactivity of cells which had been preincubated with cytochalasin B (20 µM) was subtracted from the total cell-associated radioactivity of each individual incubation mixture (Gliemann et al., 1972, *Biochim. Biophys. Acta* 286, 1-9).

#### Example 7: Lipogenesis

Lipogenesis was measured as the incorporation of D-glucose into toluene-extractable lipids (Moody et al., 1974, *Horm. Metab. Res.* 6, 12-16). 200 µl of the adipocyte suspension in KRH were incubated in scintillation vials, at 37°C for 20 min, in 680 µl of KRH which was supplemented with 3.5 mM glucose and 20 µl of insulin solution. Lipogenesis was started by adding 100 µl of D-[3-<sup>3</sup>H]glucose (25 µCi/ml KRH). After incubating at 37°C, and gently shaking under an atmosphere of 5% CO<sub>2</sub>, for 90 min, 10 ml of scintillation fluid (toluene-based) were added and the radioactivity in the toluene phase was determined after shaking vigorously and subsequent phase separation (at least 4 h of incubation). The radioactivity of the

lipids in the toluene phase was corrected for the radioactivity of an incubation mixture which contained the same quantity of [ $^3\text{H}$ ]glucose but no cells.

**Example 8: Inhibition by leptin of insulin-induced glucose transport and of insulin-induced lipogenesis**

Isolated rat adipocytes were incubated for 15 h in primary culture in the presence or absence of increasing concentrations of leptin. The cells were then washed and tested for glucose transport and lipogenesis in the basal and insulin (10 nM)-stimulated states. The stimulation factor for insulin was calculated as the ratio between insulin-stimulated and basal activities. Each value represents the mean from two independent adipocyte cultures with activity being determined two or three times in each case.

**Table 1: Insulin concentration: 5 nM**

Leptin	Glucose	
Concentration [nM]	Transport*	Lipogenesis*
0	13.4	3.9
0.3	13.4	3.9
1	11.75	3.2
3	7.4	2.15
10	3.5	1.5
30	2.35	1.15
100	1.5	1.05

\* The factors are the quotients of the values following stimulation and the basal values.

The results are summarized in Table 1.

Example 9: Influence of leptin on the insulin dose/effect curve

Isolated rat adipocytes were incubated for 16.5 h in primary culture in the presence or absence of increasing concentrations of leptin. The cells were then washed and tested for the stimulation of glucose transport by differing concentrations of insulin. Glucose transport activity is given as a "dpm value" of the 2-deoxy- $^3\text{H}$ glucose which is specifically associated with the cells. Each value represents the mean from two independent adipocyte cultures with the activity being determined four times in each case.

Table 2: Measured parameter: glucose transport

	Leptin [nM]							
Insulin [nM]	0	0.05	1	2	5	10	30	100
0	671	654	634	688	712	755	688	747
0.02	784	735	678	704	734	773	704	766
0.05	1285	1025	824	755	798	802	745	780
0.1	2406	1674	1189	860	883	856	789	803
0.2	4762	3320	1587	1006	923	941	852	813
0.5	6976	5138	2180	1377	1167	1209	943	883
1	7981	6834	3904	1916	1583	1573	1183	896
2	8576	7942	5510	2680	2140	2061	1374	1034
5	8956	8794	6985	4323	2950	2476	1782	1205
10	9064	9134	8241	6107	3682	2710	1972	1451
50	9072	9189	8932	7032	4031	2967	2114	1723

The value given is in each case the uptake of  $^3\text{H}$ -labeled 2-deoxyglucose measured in dpm (disintegrations per minute).

The results are summarized in Table 2.

Example 10: The antagonism of fragment 116-167 to the leptin effect

Isolated rat adipocytes were incubated for 17.5 h in primary culture in the presence

or absence of leptin (10 nM) and increasing concentrations of leptin fragment 116-167. The cells were then washed and tested for stimulation of glucose transport or lipogenesis by 5 nM insulin. The stimulation factor for insulin was calculated as the ratio between the insulin-stimulated activity and the basal activity. Each value represents the mean from two independent adipocyte cultures with activity being determined three times in each case.

Table 3:      Leptin: 10 nM  
                 Insulin: 5 nM

Fragment	Glucose	
Concentration [nM]	Transport*	Lipogenesis
0	3.55	1.5
0.3	3.65	1.65
1	4.5	2.15
3	5.75	2.65
10	7.75	3.1
30	10.8	3.6
100	12.55	4
300	13.2	4
Insulin only	13.4	3.9

\*      The factors are the quotients of the values following stimulation and the basal values.

The results are summarized in Table 3.

## Appendix:

Table 4: SEQ ID NO.: 4

## Human leptin

1- Met His Trp Gly Thr Leu Cys Gly Phe Leu Trp Leu Trp Pro Tyr  
 Leu Phe Tyr Val Gln Ala Val Pro Ile Gln Lys Val Gln Asp Asp  
 Thr Lys Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp Ile  
 Ser His Thr Gln Ser Val Ser Ser Lys Gln Lys Val Thr Gly Leu  
 Asp Phe Ile Pro Gly Leu His Pro Ile Leu Thr Leu Ser Lys Met  
 Asp Gln Thr Leu Ala Val Tyr Gln Gln Ile Leu Thr Ser Met Pro  
 Ser Arg Asn Val Ile Gln Ile Ser Asn Asp Leu Glu Asn Leu Arg  
 Asp Leu Leu His Val Leu Ala Phe Ser Lys Ser Cys His Leu Pro  
 Trp Ala Ser Gly Leu Glu Thr Leu Asp Ser Leu Gly Gly Val Leu  
 Glu Ala Ser Gly Tyr Ser Thr Glu Val Val Ala Leu Ser Arg Leu  
 Gln Gly Ser Leu Gln Asp Met Leu Trp Gln Leu Asp Leu Ser Pro  
 Gly Cys-167

Table 5: SEQ ID NO.: 5

## Murine leptin

1-Met Cys Trp Arg Pro Leu Cys Arg Phe Leu Trp Leu Trp Ser Tyr  
 Leu Ser Tyr Val Gln Ala Val Pro Ile Gln Lys Val Gln Asp Asp  
 Thr Lys Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp Ile  
 Ser His Thr Gln Ser Val Ser Ala Lys Gln Arg Val Thr Gly Leu  
 Asp Phe Ile Pro Gly Leu His Pro Ile Leu Ser Leu Ser Lys Met  
 Asp Gln Thr Leu Ala Val Tyr Gln Gln Val Leu Thr Ser Leu Pro  
 Ser Gln Asn Val Leu Gln Ile Ala Asn Asp Leu Glu Asn Leu Arg  
 Asp Leu Leu His Leu Leu Ala Phe Ser Lys Ser Cys Ser Leu Pro  
 Gln Thr Ser Gly Leu Gln Lys Pro Glu Ser Leu Asp Gly Val Leu  
 Glu Ala Ser Leu Tyr Ser Thr Glu Val Val Ala Leu Ser Arg Leu  
 Gln Gly Ser Leu Gln Asp Ile Leu Gln Gln Leu Asp Val Ser Pro  
 Glu Cys-167

Table 6: SEQ ID NO.: 6

## Fragment 116-167 from murine leptin

116-Ser Cys Ser Leu Pro Gln Thr Ser Gly Leu Gln Lys Pro Glu Ser  
Leu Asp Gly Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val Val  
Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Ile Leu Gln Gln  
Leu Asp Val Ser Pro Glu Cys-167

The two cysteines present in the sequences shown in Tables 4 to 6 are linked by a disulfide bridge.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

- (A) NAME: Hoechst Aktiengesellschaft
- (B) STREET: -
- (C) CITY: Frankfurt
- (D) FEDERAL STATE: -
- (E) COUNTRY: Germany
- (F) POSTAL CODE: 65926
- (G) TELEPHONE: 069-305-3005-
- (H) TELEFAX: 069-35-7175
- (I) TELEX: -

(ii) TITLE OF APPLICATION: Use of leptin antagonists for treating insulin resistance in type II diabetes

(iii) NUMBER OF SEQUENCES: 6

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURES:

- (A) NAME/KEY: exon
- (B) LOCATION: 1..20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAATGCAGAA TAAATAAATA

20

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURES:

- (A) NAME/KEY: exon
- (B) LOCATION: 1..34

## 22

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAAAGAAGGA TCCAGTGCCT ATCCAGAAAG TCCA

34

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURES:

- (A) NAME/KEY: exon
- (B) LOCATION: 1..34

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGAGAGAAGC TTGAGGGAGA GAAATGAATG ATGG

34

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 167 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURES:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..167

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```

Met His Trp Gly Thr Leu Cys Gly Phe Leu Trp Leu Trp Pro Tyr Leu
 1              5              10              15

Phe Tyr Val Gln Ala Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys
 20              25              30

Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr
 35              40              45

Gln Ser Val Ser Ser Lys Gln Lys Val Thr Gly Leu Asp Phe Ile Pro
 50              55              60

Gly Leu His Pro Ile Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala
 65              70              75              80

Val Tyr Gln Gln Ile Leu Thr Ser Met Pro Ser Arg Asn Val Ile Gln
 85              90              95

Ile Ser Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Val Leu Ala
100              105              110

Phe Ser Lys Ser Cys His Leu Pro Trp Ala Ser Gly Leu Glu Thr Leu
115              120              125

```



## 23

Asp Ser Leu Gly Gly Val Leu Glu Ala Ser Gly Tyr Ser Thr Glu Val  
 130 135 140

Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Met Leu Trp Gln  
 145 150 155 160

Leu Asp Leu Ser Pro Gly Cys  
 165

## (2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 167 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

- (ix) FEATURES:  
 (A) NAME/KEY: Peptide  
 (B) LOCATION: 1..167

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Met Cys Trp Arg Pro Leu Cys Arg Phe Leu Trp Leu Trp Ser Tyr Leu  
 1 5 10 15

Ser Tyr Val Gln Ala Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys  
 20 25 30

Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr  
 35 40 45

Gln Ser Val Ser Ala Lys Gln Arg Val Thr Gly Leu Asp Phe Ile Pro  
 50 55 60

Gly Leu His Pro Ile Leu Ser Leu Ser Lys Met Asp Gln Thr Leu Ala  
 65 70 75 80

Val Tyr Gln Gln Val Leu Thr Ser Leu Pro Ser Gln Asn Val Leu Gln  
 85 90 95

Ile Ala Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Leu Leu Ala  
 100 105 110

Phe Ser Lys Ser Cys Ser Leu Pro Gln Thr Ser Gly Leu Gln Lys Pro  
 115 120 125

Glu Ser Leu Asp Gly Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val  
 130 135 140

Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Ile Leu Gln Gln  
 145 150 155 160

Leu Asp Val Ser Pro Glu Cys  
 165

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 52 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURES:

- ```

EXPOSURES:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..52

```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Ser Cys Ser Leu Pro Gln Thr Ser Gly Leu Gln Lys Pro Glu Ser Leu  
1 5 10 15

Asp Gly Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val Val Ala Leu  
20 25 30

Ser Arg Leu Gln Gly Ser Leu Gln Asp Ile Leu Gln Gln Leu Asp Val  
35 40 45

Ser Pro Glu Cys  
50

## Patent claims:

1. A pharmaceutical composition, comprising a leptin antagonist in an amount therapeutically beneficial for the treatment of Type II diabetes.
2. A pharmaceutical composition according claim 1, wherein said leptin antagonist is derived from a human or animal leptin.
3. A pharmaceutical composition according to claim 2, wherein said leptin antagonist is derived from a leptin which is selected from the group consisting of human, rat, mouse, pig and anthropoid ape leptins.
4. A pharmaceutical composition according to claim 3, wherein said leptin antagonist is derived from murine leptin.
5. A pharmaceutical composition according to claim 4, wherein said leptin antagonist is either an internal or carboxy-terminal fragment of murine leptin.
6. A pharmaceutical composition according to claim 5, wherein said leptin antagonist is a murine leptin fragment comprising the amino acid sequence of Table 6 (SEQ ID NO.: 6).
7. A pharmaceutical composition according to claim 6, wherein said leptin antagonist contains one or more conservative amino acid substitutions.
8. A pharmaceutical composition according to claim 7, wherein said conservative amino acid substitution either exchanges one ionic amino acid for another ionic amino acid or exchanges one hydrophobic amino acid for another hydrophobic amino acid.
9. A pharmaceutical composition according to claim 8, wherein said conservative amino acid substitution is selected from the group consisting of aspartate for glutamate substitutions, glutamate for aspartate substitutions,

leucine for isoleucine substitutions, and isoleucine for leucine substitutions.

10. A pharmaceutical composition according to claim 6, wherein said leptin antagonist is modified by a chemical modification which is selected from the group consisting of acetylation, carbamoylation, formylation, biotinylation, acylation, derivatization with polyethylene glycol and derivatization with hydrophilic polymers.
11. A pharmaceutical composition according to claim 1, wherein said leptin antagonist is selected from the group consisting of a soluble leptin receptor, a leptin receptor fragment, a fusion with a soluble leptin receptor, and a fusion with a leptin receptor fragment.
12. A method of treating Type II diabetes, comprising administering to a patient in need of said treatment a therapeutically effective amount of a composition according to claim 1.
13. A method according to claim 12, wherein said composition comprises a leptin antagonist which is a murine leptin fragment comprising the amino acid sequence of Table 6 (SEQ ID NO.: 6).
14. A method according to claim 13, wherein said leptin antagonist contains one or more conservative amino acid substitutions.
15. A method according to claim 12, wherein said composition comprises a leptin antagonist selected from the group consisting of a soluble leptin receptor, a leptin receptor fragment, a fusion with a soluble leptin receptor, and a fusion with a leptin receptor fragment.
16. A method of restoring or amplifying the physiological effect of insulin, comprising administering to a patient in need of said restoration or amplification a therapeutically effective amount of a composition according to claim 1.

17. A method according to claim 16, wherein said composition comprises a leptin antagonist which is a murine leptin fragment the amino acid sequence of Table 6 (SEQ ID NO.: 6).
18. A method according to claim 17, wherein said leptin antagonist contains one or more conservative amino acid substitutions.
19. A method according to claim 16, wherein said composition comprises a leptin antagonist selected from the group consisting of a soluble leptin receptor, a leptin receptor fragment, a fusion with a soluble leptin receptor, and a fusion with a leptin receptor fragment.
20. A murine leptin fragment which comprises amino acids 116 to 167 or 116 to 166 of murine leptin.
21. A process for the preparation of a leptin fragment according to any of the claims 2-11 and 20, wherein
  - (a) a gene encoding said leptin fragment is expressed making use of a suitable vector/host system; and
  - (b) said leptin fragment is isolated from the host system.

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 97/05035

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C07K14/575 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                          | Relevant to claim No.      |
|------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------|
| X          | WO 96 05309 A (UNIV ROCKEFELLER ; FRIEDMAN JEFFREY M (US); ZHANG YIYING (US); PROE)<br>22 February 1996<br>*sequence 20*<br>see claims 1,2,14,61,63,73<br>---                                                                                                               | 1-9,12,<br>16,20           |
| X          | KIEFFER ET AL.: "Leptin receptors Expressed on Pancreatic beta- Cells"<br>BIOCHEM.BIOPHYS.RES.COMMUN.,<br>vol. 224, no. 2, July 1996,<br>pages 522-27, XP002054912<br>see page 524, line 3 - line 5<br>see page 525, line 8 - line 9<br>see page 525, last paragraph<br>--- | 1-4,11,<br>12,15,<br>16,19 |
| P,X        | WO 97 26335 A (UNIV ROCKEFELLER) 24 July 1997<br>see claims 1,8,9,63,65<br>-----                                                                                                                                                                                            | 11,15,19                   |

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

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- \*E\* earlier document but published on or after the international filing date
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- \*A\* document member of the same patent family

Date of the actual completion of the international search

6 February 1998

Date of mailing of the international search report

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 97/05035

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
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|                                           |                     | BG 101228 A                | 30-09-97            |
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|                                           |                     | ZA 9506868 A               | 09-04-96            |
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| WO 9726335 A                              | 24-07-97            | AU 2244797 A               | 11-08-97            |
| -----                                     |                     |                            |                     |